Express Mail No: EV312711638US
Date of Deposit: 10/27/04

Attorney Docket No: 23239-301B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Ellington, *et al.*		
SERIAL NUMBER:	09/666,870	EXAMINER:	Jon D. Epperson, Ph.D.
FILING DATE:	September 20, 2000	ART UNIT:	1639
FOR:	METHOD AND APPARATUS FOR IDENTIFYING ALLOSTERICALLY REGULATED RIBOZYMES		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF PRIOR INVENTION UNDER 37 C.F.R. § 1.131

We, Andrew Ellington, Michael Robertson, J. Colin Cox, Timothy Riedel and Eric Davidson, hereby declare and state as follows:

1. We are aware that in the final Office Action dated April 28, 2004 in the above-identified application ("the Application"), the Examiner has rejected claims 47, 49, 54-59 and 61-66 under 35 U.S.C. § 102(a) as being anticipated by Marshall and Ellington, Nature Structural Biology, 6(11):992-94 (1999) ("Marshall"). We are also aware that the Examiner has rejected claims 47, 49, 54, 58, 61-62 and 65-66 under 35 U.S.C. §102(a) as being anticipated by Hesselberth *et al.*, Reviews in Molecular Biotechnology, 74:15-25 (2000) ("Hesselberth"). This Declaration is to establish that the Marshall and Hesselberth publications do not describe an invention that was known or used by others before the invention of the subject matter recited in the claims of the instant Application under 35 U.S.C. § 102(a).

2. We, Andrew Ellington, Michael Robertson, J. Colin Cox, Timothy Riedel and Eric Davidson, are the named inventors in this case. We note that Andrew Ellington is the Principal Investigator of the laboratory where the subject matter recited in the claims of the instant Application was invented.

3. This Application is based in part on work that was reported in two manuscripts that subsequently published as two of the references the Examiner has cited against the Application, *i.e.*, the Marshall and Hesselberth references.

The Marshall reference is not prior art under 35 U.S.C. § 102(a)

4. The work presented in Figure 3 on page 993 of the Marshall reference, and at page 994, first full paragraph, corresponds to the description provided at page 59, line 21 through page 61, line 11 and in Figures 16a, 16b and 16c of the as-filed Application.

5. The Marshall reference describes the potential use of aptazymes that have been mounted in arrays, similar to "DNA chips", in monitoring the presence and concentrations of different analytes, such as metabolites or proteins.

6. Figure 3 of the Marshall reference depicts an example of a proposed aptazyme chip, and the description in the legend of Figure 3 describes the use of such proposed aptazyme chips in arrays to monitor the presence and concentrations of different metabolites or proteins (*i.e.*, analytes). As seen in the top panel of Figure 3, different aptazymes (*i.e.*, a heterogeneous mixture of aptazymes) can be immobilized on beads in wells, and a mixture of analytes and fluorescently tagged substrates can be added to each well. An example of a proposed aptazyme reaction is depicted in the middle panel of Figure 3. In the aptazyme reaction depicted in Figure 3, the aptazymes will covalently attach the fluorescent tags to themselves in the presence of cognate effectors. If the aptazymes are not activated by effectors, the tagged substrates will be washed out of the well during the washing step. Marshall describes the benefits of this proposed aptazyme reaction in the legend of Figure 3: "the covalent immobilization of reporters (as opposed to non-covalent immobilization techniques such as in an ELISA) should allow extremely stringent wash steps to be employed." As shown in the last panel of Figure 3, after the aptazyme reaction and the stringent washing steps, the presence and amounts of co-immobilized fluorescent tags will be indicative of the amount of ligand(s) present during the reaction.

7. These proposed aptazyme chips and methods described in the Marshall reference (*e.g.* at Figure 3, and at page 994) are the same as the inventions described and claimed in this Application. Figures 16a, 16b and 16c), schematically depict aptazyme arrays identical to

those presented in Figure 3 of Marshall. Likewise, the methods of detecting the presence and amounts of one or more analytes in a sample using the aptazyme arrays of Figures 16a, 16b and 16c are the same as the methods described by Marshall. In fact, the Marshall publication was cited in the as-filed specification at page 60, lines 18-19 as support for the methods and compositions of the claimed invention.

8. For example, the instant Application describes methods of anchoring aptazymes to various substrates by immobilizing the aptazymes on beads in wells of a plate or by covalently attached the aptazyme to a substrate, such as, for example, glass or silicon. (*See* specification at page 59, line 21 through page 60, line 8 and at page 60, line 22 through page 61, line 2).

9. The instant Application also discloses use of these anchored aptazymes to detect the presence and amounts of particular analytes in a sample at page 60, lines 7-19. In particular, this passage in the instant Application teaches that different ribozyme ligases are immobilized on beads in wells of a plate, and mixtures of analytes and tagged substrates are added to each well. In the presence of cognate effectors, the aptazymes will "covalently attach the reporter tags (*e.g.*, fluorescent tags) to themselves," and "where aptazymes have not been activated by effectors, the tagged substrates are washed out of the well." After the reaction and washing steps, "the presence and amounts of co-immobilized reporter tags are indicative of amounts of ligands that were present during the reaction."

10. Thus, the methods and arrays described by the Marshall reference are precisely the invention disclosed and claimed in this Application, and the Marshall reference describes the work that ultimately produced the aptazymes, arrays and methods of our claimed invention. Accordingly, the inventions claimed in this Application were necessarily invented before the publication date of the Marshall reference. Therefore, the methods and compositions recited by the pending claims were not known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before we invented the claimed inventions. As such, Marshall is not prior art under 35 U.S.C. § 102(a).

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11. The work presented at page 24, col. 1, last full paragraph through col. 2, first full paragraph of the Hesselberth reference corresponds to the description provided at page 59, line 21 through page 61, line 11 of the as-filed Application.

12. The Hesselberth reference describes the potential use of aptamers and aptazymes to generate signals. For example, Hesselberth discloses that aptazyme ligases could be used to ligate an oligonucleotide containing a fluorescent dye to themselves. (*See* Hesselberth at p. 24, col. 1, lines 39-44). Hesselberth also describes that any number of reporter tags could be used including, for example, oligonucleotides coupled to enzymes that turnover fluorescent substrates and oligonucleotides that generate electrons for electrochemical sensing, such as horseradish peroxidase. (*See* Hesselberth at p. 24, col., lines 1-3).

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14. While the Hesselberth reference describes potential examples of aptazyme arrays, this reference explicitly states at p. 25, col. 2, lines 24-25 that the named authors, including Dr. Ellington and Dr. Robertson, "are currently working towards proofs-of-principle for such arrays." These proposed arrays alluded to in the Hesselberth reference ultimately developed into the arrays (and methods of use thereof) disclosed and claimed in the instant Application, *e.g.*, at page 59, line 21 through page 61, line 11.

15. The Application describes aptamers and aptazymes that have been modified to generate detectable signals are described at page 60, lines 7-19. In particular, this passage of the instant Application discloses the use of aptazyme ligases (also referred to in the instant application as "ribozyme ligases") that covalently attach reporter tags, such as fluorescent tags, to themselves in the presence of effector molecules. At page 61, lines 3-11, the Application describes how the covalent attachment of reporter tags allows for the use of stringent wash steps, without destroying signal. The Application also describes that in the aptazyme arrays of the instant invention, "different ribozyme ligases" (also referred to in the instant Application as aptazymes) are immobilized on different beads in wells or directly attached to a solid support or substrate (*e.g.*, by a covalent bond). (*See e.g.*, p. 60, lines 7-9; p. 61, lines 1-2). Thus, the aptazyme arrays described and claimed in the instant Application detect the presence and amount of these covalently attached reporter tags, which, in turn, act as indicators of the amount of ligand present in a particular sample.

16. Thus, the methods and arrays proposed by the Hesselberth reference are precisely the invention disclosed and claimed in this Application, and in fact, the Hesselberth reference describes some of our work that ultimately produced the aptazymes, arrays and methods of our claimed invention. Accordingly, the inventions claimed in this Application were necessarily invented before the publication date of the Hesselberth reference. Therefore, the methods and compositions recited by the pending claims were not known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before we invented the claimed inventions. As such, Hesselberth is not prior art under 35 U.S.C. § 102(a).

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19. As a person signing below, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Andrew Ellington — 10/21/04

Declarant's Signature — Date
Full Name of Declarant: Andrew D. Ellington

Declarant's Signature — Date
Full Name of Declarant: Michael P. Robertson

Declarant's Signature — Date
Full Name of Declarant: J. Colin Cox

Declarant's Signature
Full Name of Declarant: Timothy E. Riedel

Oct. 25th, 2004
Date

Declarant's Signature
Full Name of Declarant: Eric A. Davison

10/22/2004
Date

Attachments:
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_____ _____

Declarant's Signature Date

Full Name of Declarant: Andrew D. Ellington

Michael Robertson 10-6-04

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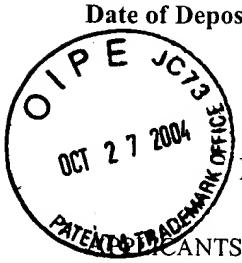

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12. The Hesselberth reference describes the potential use of aptamers and aptazymes to generate signals. For example, Hesselberth discloses that aptazyme ligases could be used to ligate an oligonucleotide containing a fluorescent dye to themselves. (*See* Hesselberth at p. 24, col. 1, lines 39-44). Hesselberth also describes that any number of reporter tags could be used including, for example, oligonucleotides coupled to enzymes that turnover fluorescent substrates and oligonucleotides that generate electrons for electrochemical sensing, such as horseradish peroxidase. (*See* Hesselberth at p. 24, col., lines 1-3).

13. At p. 24, col. 2, lines 4-28, the Hesselberth reference describes the possible production of arrays having different aptamers and aptazymes covalently immobilized on a chip surface. In one example of such a potential array, "aptazymes could be immobilized and analytes and oligonucleotides tags introduced together," such that the presence of an analyte mixture would activate "specific aptamers in specific sectors to pull down specific tags, thereby allowing both spatial and spectral resolution of the analyte detection." (*See*, Hesselberth, p. 24, col. 2, lines 11-24). Like the Marshall reference (*e.g.*, at Figure 3), Hesselberth also suggests that after the proposed aptazyme reaction, the reporter tags will be covalently attached to the aptazymes, and the aptazymes, in turn, will be covalently attached to the chip surface, thereby allowing the aptazyme chips to be "stringently washed" to reduce non-specific binding and background.

14. While the Hesselberth reference describes potential examples of aptazyme arrays, this reference explicitly states at p. 25, col. 2, lines 24-25 that the named authors, including Dr. Ellington and Dr. Robertson, "are currently working towards proofs-of-principle for such arrays." These proposed arrays alluded to in the Hesselberth reference ultimately developed into the arrays (and methods of use thereof) disclosed and claimed in the instant Application, *e.g.*, at page 59, line 21 through page 61, line 11.

15. The Application describes aptamers and aptazymes that have been modified to generate detectable signals are described at page 60, lines 7-19. In particular, this passage of the instant Application discloses the use of aptazyme ligases (also referred to in the instant application as "ribozyme ligases") that covalently attach reporter tags, such as fluorescent tags, to themselves in the presence of effector molecules. At page 61, lines 3-11, the Application describes how the covalent attachment of reporter tags allows for the use of stringent wash steps, without destroying signal. The Application also describes that in the aptazyme arrays of the instant invention, "different ribozyme ligases" (also referred to in the instant Application as aptazymes) are immobilized on different beads in wells or directly attached to a solid support or substrate (*e.g.*, by a covalent bond). (*See e.g.*, p. 60, lines 7-9; p. 61, lines 1-2). Thus, the aptazyme arrays described and claimed in the instant Application detect the presence and amount of these covalently attached reporter tags, which, in turn, act as indicators of the amount of ligand present in a particular sample.

16. Thus, the methods and arrays proposed by the Hesselberth reference are precisely the invention disclosed and claimed in this Application, and in fact, the Hesselberth reference describes some of our work that ultimately produced the aptazymes, arrays and methods of our claimed invention. Accordingly, the inventions claimed in this Application were necessarily invented before the publication date of the Hesselberth reference. Therefore, the methods and compositions recited by the pending claims were not known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before we invented the claimed inventions. As such, Hesselberth is not prior art under 35 U.S.C. § 102(a).

The Cox reference

17. We are also aware that the Examiner has cited the following publication: Cox *et al.*, Biotechnol. Prog., 14:845-50 (1998) ("Cox") under 35 U.S.C. § 103(a). We note that the this publication represents earlier efforts by Dr. Ellington and Dr. Cox to produce an automated *in vitro* selection procedure for generating novel nucleic acid aptamers.

18. The Cox reference does not describe or suggest automated methods for detecting an aptazyme reaction. At the time the Cox reference was published, we had not yet invented aptazyme arrays, or methods of using such arrays to detect an aptazyme reaction. Therefore, this reference is insufficient to lead one of ordinary skill in the art to produce the methods and arrays of the claimed invention.

19. As a person signing below, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____ _____
Declarant's Signature Date
Full Name of Declarant: Andrew D. Ellington

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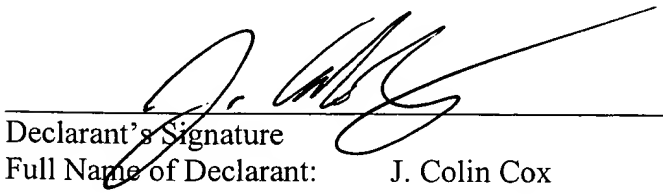
Declarant's Signature Date
Full Name of Declarant: Michael P. Robertson



10/4/2004
Declarant's Signature Date
Full Name of Declarant: J. Colin Cox

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Declarant's Signature Date

Full Name of Declarant: Timothy E. Riedel

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Declarant's Signature Date

Full Name of Declarant: Eric A. Davison

Attachments:

Marshall *et al.*, Nature Structural Biology, 6(11):992-94 (1999)

Hesselberth *et al.*, Reviews in Molecular Biotechnology, 74:15-25 (2000)

Cox *et al.*, Biotechnol. Prog., 14:845-50 (1998)

TRA 1962093v1

Training ribozymes to switch

Kristin A. Marshall and Andrew D. Ellington

Ribozymes that are sensitive to cyclic nucleotides have been selected *in vitro*. Remarkably, the cGMP-dependent ribozymes are specifically activated by a factor of 5,000 — the largest allosteric ribozyme activation by a small molecule seen to date.

In 1985, Norman Pace predicted that one of the advantages of ribozyme catalysts relative to protein catalysts would be their greater flexibility[1]. At the time, this prediction was difficult to credit, since it was already known that the catalytic activities of many proteins were modulated by allosteric effectors. In the interim, the examination of the structures and mechanisms of natural ribozymes suggested that dynamic motions indeed contributed to catalysis[2]. However, the full extent to which conformational changes can influence ribozyme catalysis is only now becoming apparent from the study of non-natural ribozymes. On page 1062 of this issue of *Nature Structural Biology*, Ronald Breaker and coworkers[3] at Yale University demonstrate that selected ribozymes can have remarkable allosteric responses that are orders of magnitude greater than those typically seen for protein enzymes.

Breaker and coworkers had previously shown that ribozyme catalysis could be modulated by small, allosteric effectors. They added an anti-adenosine aptamer to a non-essential stem of the hammerhead ribozyme, and the resultant chimera (which we call an aptazyme[4]) was down-regulated by 180-fold by ATP[5]. An investigation of the mechanism showed that a ligand-induced conformational change in the aptamer[6] caused a steric clash between the aptamer and the hammerhead[7]. Other constructs in which the connecting stems between aptamers and ribozymes were designed to be weak and relatively unstructured in the absence of ligands, but strengthened in their presence, led to allosteric activation[8]. Breaker's group has since built upon these feats by randomizing the connecting regions between aptamers and hammerhead, and then selecting so-called 'communication modules' that facilitate ligand-dependent increases or decreases in catalytic activity[9]. Remarkably, different aptamers could be joined to the hammerhead via the same communication modules and could change which ligand controls catalysis.

This issue of *Nature Structural Biology* presents a logical progression in the story, the selection of ligand-dependent hammerheads from a pool in which the allosteric domain has been completely randomized. In this respect, the selection of aptazymes has now been put on a footing similar to the selection of their namesake aptamers, nucleic acid binding species isolated from random sequence pools (Fig. 1). A pool of 25 random nucleotides was introduced at the termini of hammerhead stem-loop II, in much the same position as various aptamers been appended[5,8,9]. The random region was joined to the ribozyme by a communication module that had been obtained in a selection for flavin activation[9]. Effector-dependence was enriched during the selection by using gel electrophoresis to first remove ribozymes from the population that could cleave themselves in the absence of effector, then isolating and preferentially amplifying ribozymes that could cleave themselves in the presence of effector (Fig. 1). Based on previous results[9] it was likely that this strategy would work. Therefore, to further increase the complexity (and interest!) of the experiment, a pool of effectors, the four cyclic nucleoside monophosphates, was incubated with the pool of ribozymes. After 14 cycles of coupled negative and positive selections, the pool showed a slight ability to cleave more rapidly in the presence of cGMP (Fig. 2). The population continued to improve in the presence of cGMP, so a new hurdle was set by including cGMP in the negative selection for cycle 17 and concomitantly omitting it from the positive selection. The pool lost its cGMP-dependence and instead became cCMP-activated at generation 19. Cyclic CMP was then removed, and the pool became cAMP dependent at generation 22. A final six rounds of selection were carried out with cUMP as the sole remaining effector, but no cUMP-dependent ribozymes were ever obtained. The

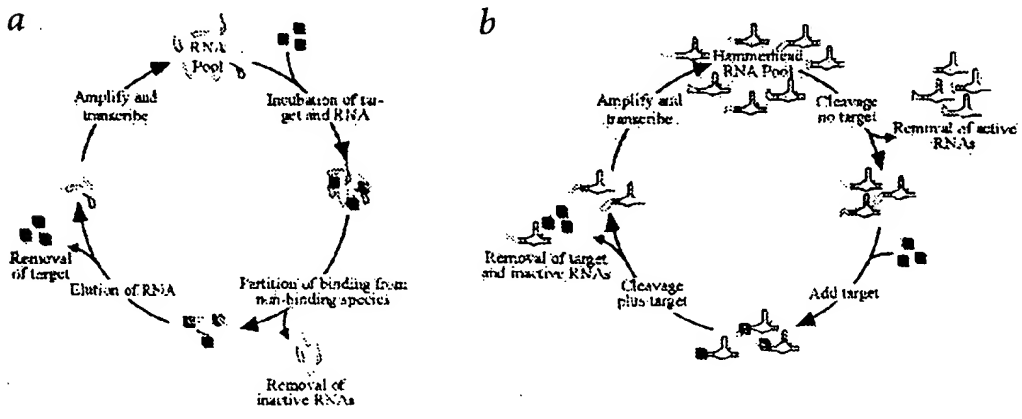

Fig. 1 Aptamer selection *versus* aptazyme selection. ***a***, Binding species (aptamers) can be iteratively selected from random sequence pools via co-immobilization with a ligand (left). ***b***, In contrast, aptazymes can be selected directly in solution without co-immobilization based on the effector-dependent modulation of catalysis (in the example shown, activation of cleavage). Because of this, the allosteric domains of aptazymes may eventually be found to enclose their targets more completely than the binding sites of aptamers.

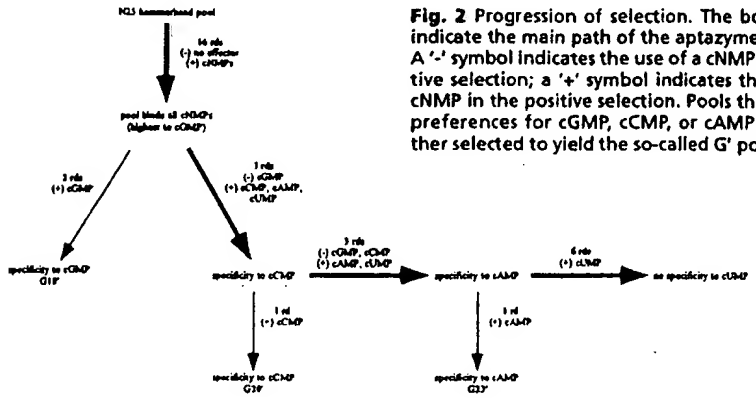

Fig. 2 Progression of selection. The bold arrows indicate the main path of the aptazyme selection. A '-' symbol indicates the use of a cNMP as a negative selection; a '+' symbol indicates the use of a cNMP in the positive selection. Pools that showed preferences for cGMP, cCMP, or cAMP were further selected to yield the so-called G' pools.

different pools that were dependent on different cyclic nucleotides were further selected to strengthen that dependence.

These selection experiments are notable not just for their temerity and novelty, but because the aptazymes that have been created have extraordinary properties. Clones from each of the final pools (G18', G20' and G23'; Fig. 2) could not only discriminate between different cNMPs, but could also preferentially recognizes a single cNMP over the corresponding NMP or modified cNMPs. However, the selected aptazymes were at least partially active in the presence of corresponding nucleosides or 2'-deoxy nucleosides. The specificities of the allosteric domains of these aptazymes thus appear to be quite close to that of aptamers, indicating that it may be possible to freely select binding domains using catalysts rather than affinity columns. The aptazymes also exhibited the highest activation of any published to date. Rational design experiments from Breaker's group have produced an ATP-dependent hammerhead that shows 10-fold activation[5] while our own rational design experiments have yielded an ATP-dependent ligase that shows 830-fold activation[4] and a theophylline-dependent ligase that shows 1,600-fold activation[10]. Communication module selections generated an flavin mononucleotide-dependent (FMN-dependent) hammerhead that shows 270-fold activation[9]. In contrast, the best cNMP-dependent aptazymes derived from the pool containing a completely random allosteric domain showed 5000-fold activation. The existence of these non-natural ribozymes with unprecedented dynamic properties should prove a great boon to structural biologists, who have either solved or made great strides towards solving the structures of all the 'big 8' natural catalysts (hammerhead, hairpin, HDV ribozyme, VS ribozyme, Group I intron, Group II intron, RNAse P, and ribosomal RNA).

What is perhaps most surprising about these selections, however, is that they were successful at all. After six rounds of selection, the pool showed an increase in activation when the cNMP effectors were added, but apparently could not specifically recognize any individual cNMP. Instead, it was discovered that the pool was responsive to pH changes that occurred upon the cNMP addition. Nonetheless, the researchers forged ahead, assuming that the desired molecules were still interspersed among the selected pH indicators (an interesting sub-class that can apparently "remember acid pulses"; R. Breaker, pers. comm.). In addition, two classes of 'selfish' RNAs plagued the selection. One mundane class of survivors had reduced catalytic rates in both the negative and positive reactions; these enzymes were just slow enough to survive the negative purge and still show up in the positive selection. A second, somewhat more interesting class slowly converted from misfolded to properly folded conformers. Against the backdrop of both unwanted 'successes' the researchers again forged ahead. The fact that cNMP-activated aptazymes were eventually selected is a stirring testament to the raw power of negative selections and fast, iterative techniques. Indeed, it was likely far harder to remove the numerous, freeloaders that accumulated during the selection than the preponderance of neutral species that populated the naive pool. Even the design of the pool may have worked against Breaker's group. The sequences of the final aptazymes revealed that the built-in communication module that was supposed to encourage interactions between allosteric domain and ribozyme had been extensively mutated. The ability of selection to override design has been previously observed in the mutation of cofactor-binding domains in selected ribozyme kinases[11] and the generation of alternative substrate-binding domains in selected ribozyme ligases[12].

Most importantly, though, these results now set the stage for the utilization of aptazymes as generalized reagents for signal transduction. To the extent that random sequence allosteric domains can bring forth a variety of aptazymes, and to the extent that selections can be carried out in parallel against pools of effector

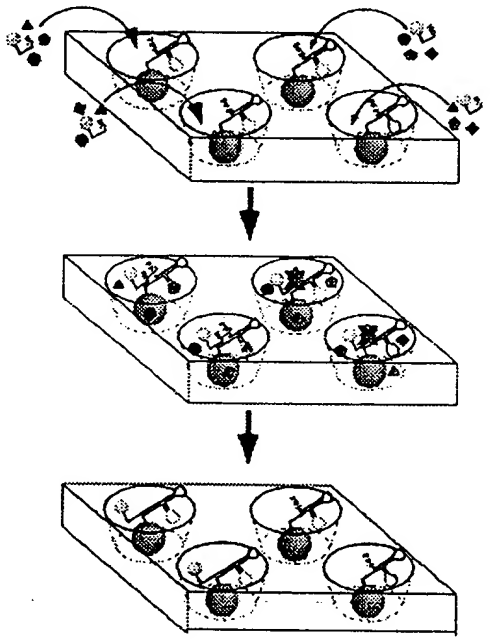

Fig. 3 Aptazyme chips. Aptazymes can potentially be mounted in arrays and used to monitor the presence and concentrations of different metabolites or proteins (analytes). In the uppermost panel, different ribozyme ligases (indicated by different colored allosteric sites) are shown immobilized on beads in wells and mixtures of analytes (differentiated by shape and color) and fluorescently tagged substrates have been added to each well. In the middle panel, where cognate effectors are present (same color analyte and allosteric site), the aptazymes will covalently attach the fluorescent tags to themselves. Where aptazymes have not been activated by effectors, the tagged substrates are washed out of the well. One advantage of this scheme is that covalent immobilization of reporters (as opposed to non-covalent immobilization, such as in an ELISA) should allow extremely stringent wash steps to be employed. In the last panel, after reaction and washing, the presence and amounts of co-immobilized fluorescent tags are indicative of the amounts of ligands that were present during the reaction.

molecules, it should now be possible to generate large numbers of aptazymes against large numbers of effectors. Indeed, aptamers have been selected against targets that range from zinc to whole organisms[13,14] and there is no reason to believe that the recognition abilities of aptazymes will be any less.

The transformation of molecular recognition directly to molecular catalysis affords a variety of opportunities for signal generation. For example, hammerhead aptazymes could potentially cleave quenchers away from fluors. Similarly, ligase aptazymes could potentially co-immobilize oligonucleotides bearing a variety of reporters, from fluors to enzymes to magnetic particles. Interestingly, aptazyme ligases have the unique property of being able to transduce effectors into templates that can be amplified, affording an additional boost in signal prior to detection[4]. Aptazymes can be mounted in arrays using some of the same technologies that have been used to create 'DNA chips' but could instead be used to monitor the presence and concentrations of different metabolites or proteins, rather than mRNAs (Fig. 3). Finally, the new frontier for aptazymes will be *in vivo*, where they can potentially be used to develop programmed genetic circuits that can be used to regulate gene expression. In particular, aptazymes may abet the development of exquisitely regulated gene therapies.

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Folding alphabets

Hue Sun Chan

A new computational approach optimizes searches for reduced protein folding alphabets that use fewer than 20 types of amino acids. The predicted optimal five-letter alphabet happens to be in agreement with the suggestive results of a recent experiment, but whether highly reduced alphabets are sufficient for truly protein-like properties remains an open experimental question.

The prospect of using a reduced alphabet to achieve protein-like properties is appealing. There are a number of reasons for this, not the least of which is an intriguing suggestion that primordial forms of life might have once operated on a reduced alphabet[1]. In addition, it has been thought that for polypeptide chains consisting of fewer than 20 letters (that is, the 20 types of common amino acids), the physics and chemistry may be sufficiently simplified for a thorough understanding of the protein folding code. However, as has been emphasized by recent theoretical considerations (reviewed in ref. 2), a certain threshold of heterogeneity or diversity in interaction energies must be present for the polypeptides to have protein-like properties. This requirement is intuitive, as one-letter homopolymers[3] do not have unique native structures like proteins. Experimental choices of reduced alphabets, with three or more letters, have been made with this general criterion in mind. Is it possible to be more systematic in selecting reduced alphabets? On page 1033 of this issue of *Nature Structural Biology*, Wang and Wang[4] propose an algorithm for this purpose.

To arrive at an optimized reduced alphabet of size N, Wang and Wang's program of reduction first divides the 20 amino acid types into N groups, with the aim of picking a representative from each group for the final reduced alphabet. In the end, all possible groupings with N groups are considered. The best grouping is selected by a 'minimal mismatch' principle, which ensures that all interactions between amino acids belonging to any two given groups are as similar to one another as possible. If amino acids with very different properties were placed together within one group, the resulting groupings would have high levels of mismatch. Such groupings are undesirable because a significant amount of interaction heterogeneity would be lost when only one amino acid is selected from each group for the final alphabet. Wang and Wang's procedure disfavors such occurrences by minimizing mismatch. The main tenets of their proposal are given in Fig. 1. Reduction of the 20-letter alphabet entails more involved combinatorial procedures and extensive Monte Carlo sampling[4], but the principles are basically the same. One of the optimally reduced sets predicted by Wang and Wang's efforts is a five-letter alphabet: Ile, Ala, Gly, Glu, Lys (IAGEK).

Experimental interest in reduced alphabets has existed for decades. A study in 1967 found many α-helices in soluble random polypeptides of Ala, Glu, and Lys (AEK)[5]. Another study by Rao *et al.*[6] in 1974 indicated that random AEK sequences can collapse to compact, globular conformations with helical content of 46% or higher, although no individual sequence was identified. Recent years have witnessed an increase in research efforts based on the reduced-alphabet theme; the following are some examples.

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Reviews in Molecular Biotechnology 74 (2000) 15–25

Molecular Biotechnology

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Review article

In vitro selection of nucleic acids for diagnostic applications

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Accepted 13 December 1999

Abstract

In vitro selection methods have proven to be extraordinarily adept at generating a wide variety of nucleic acid-binding species (aptamers) and catalysts (ribozymes). To date, selected nucleic acids have primarily been of academic interest. However, just as antibodies have proven utility as 'universal receptors' that can be crafted against a huge variety of ligands and can be readily adapted to diagnostic assays, aptamers may yet find application in assays. A new class of research reagents, aptazymes, are not mere mimics of antibodies but in fact allow the direct transduction of molecular recognition to catalysis. Aptamers and aptazymes may prove to be uniquely useful for the development of chip arrays for the detection and quantitation of a wide range of molecules in organismal proteomes and metabolomes.

Keywords: Aptamer; Aptazyme; Signaling aptamer; Diagnostics; In vitro selection

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1389-0352/00/$ - see front matter
PII: S1389-0352(99)00005-7

1. In vitro selection

Numerous reviews have been written about the practice and results of in vitro selection (Conrad et al., 1996; Osborne et al., 1997; Famulok, 1999). For clarity, relevant procedures will be briefly reviewed here. Nucleic acid populations can be conveniently generated by chemical synthesis. The length of a random sequence tract ranges from 20 to over 150 in a single, synthetic oligonucleotide, and can be even longer if multiple, random oligonucleotides are combined into a single pool by ligation or other methods (Bartel and Szostak, 1993). The number of individuals in a random sequence population is typically at least 10^{13} and can easily be over 10^{15}. For most pools, this means that upwards of all possible 25-mers are present, and a proportionately smaller number of motifs longer than 25. Because of the redundancy of biological sequences, the sequence diversity of most random sequence pools likely rivals the sequence diversity of the Earth's biosphere.

Random sequence oligonucleotides typically contain constant regions at their 5′ and 3′ ends that facilitate enzymatic manipulations. In particular, the chemistry of a random sequence population can be manipulated during template-directed copying. For example, a nascent, single-stranded random sequence DNA oligonucleotide can be converted to a double-stranded DNA pool, which can in turn be converted to a single-stranded RNA pool (if an appropriate promoter sequence has been included in one of the constant regions) or to a single-stranded DNA pool (if biotin has been included in one of the primers). Modified nucleotides, such as phosphorothioates or ribotides with alternative chemistries at the 2′ position, can be introduced into pools, augmenting the stabilities, structures, and functionalities of individuals. A wider variety of chemistries can be appended to primers and subsequently incorporated into DNA pools.

Nucleic acid selections are geared towards isolating individuals with particular attributes from the random sequence population. For example, aptamers can be isolated by co-immobilization of individuals with a target ligand, either by filtration, immunoprecipitation, affinity chromatography, panning, or electrophoretic mobility-shift. Ribozymes can be similarly isolated, except instead of non-covalent co-immobilization researchers can select for a change in covalent immobilization. For example, ribozymes that covalently append tags to themselves, such as biotin or an oligonucleotide, can be selected from random sequence pools (Wilson and Szostak, 1995; Robertson and Ellington, 1999a). The ribozymes with appended tags can then be captured on solid supports, or in the case of oligonucleotide tags, be preferentially amplified. Alternatively, ribozymes that detach themselves from solid supports or surfaces via cleavage reactions can be selected for. In both instances, though, the selection procedure optimizes single turnover reactions. This may be one of the reasons that selected ribozymes have tended to be much slower than their protein counterparts (even the fastest known ribozyme, the Class I Bartel ligase, has a multiple turnover rate of only 100/min). Of course, it is also possible that ribozymes are much slower than their protein counterparts because of the limited repertoire of functional groups available to ribozymes. These hypotheses will likely be resolved in the future as more selections with modified RNA pools are carried out and as methods for the selection of multiple turnover ribozymes (e.g. FACS) are developed.

2. Aptamers as substitutes for antibodies

To the extent that aptamers can be thought of as universal binding agents in much the same way that antibodies have proven to be, it is obviously of interest to determine to what extent aptamers might be utilized as antibody substitutes or replacements. We originally attempted the rather simple experiment of merely radiolabeling aptamers and using them to quantitate the presence of a protein target in a cell extract (Conrad and Ellington, 1996). The anti-protein kinase C aptamers not only gave reproducible, quantitative analyses of protein concentrations, but could easily distinguish between the beta 2 isozyme against

which they were originally selected and the highly related alpha isozyme (Conrad et al., 1994). Researchers at the company NeXstar carried out similar but much more challenging experiments in which aptamers were used in place of primary antibodies in an ELISA-like assay dubbed an ELONA (enzyme-linked oligonucleotide assay) (Drolet et al., 1996). ELISA and ELONA assays were compared based on their abilities to quantitate the amount of a VEGF isoform in human serum samples. The ELONA assay yielded reproducible results very similar to those seen in a typical ELISA for VEGF. The aptamer used was not cross-reactive with other cytokines within the assay, and the concentration of the VEGF isoform could be determined down to 25 pg/ml. The assay had a dynamic range of over three orders of magnitude.

Many of the analytical techniques and refinements that have been developed around antibodies can be adapted to aptamers. For example, capillary electrophoresis has proven to be a robust and extremely sensitive technique when coupled with laser-induced fluorescence (CE-LIF). The rapid nature of the analysis (< 60 s per sample) also makes CE-LIF a lucrative alternative to other, similar diagnostic assays. Robert Kennedy and his colleagues interfaced previously selected aptamers with CE-LIF to detect IgE and thrombin in solution (German et al., 1998). The aptamer-based assay had an astounding sensitivity, with a mass detection limit of 37 zmol of IgE, and a dynamic range of 10^5. The authors could also assess IgE levels in human serum. Similarly, flow cytometry has been used to identify aptamer: elastase interactions in solution (Davis et al., 1996). The authors speculate that this system would be useful for the detection of intracellular proteins within cells, but diagnostic applications can easily be imagined in which aptamers could be used to localize and quantitate levels of a particular surface protein in a mixture of cells. Finally, simply appending biotin to an aptamer enabled the use of electrochemiluminescence (ECL) detection in a sandwich assay format (Bruno and Kiel, 1999).

3. Signaling aptamers

While there are a variety of ways in which aptamers might conveniently substitute for antibodies, the binding constants of aptamers are consistently lower than those of antibodies and the cost of aptamer production will in most cases likely exceed the cost of antibody production.

However, there are unique ways in which aptamers might be adapted to diagnostic applications that are not available to antibodies (Bier and Furste, 1997; Osborne et al., 1997). As an example, while numerous antibody conjugates to fluors and reporter enzymes have been generated, it is much simpler to site-specifically label an aptamer via chemical synthesis than to similarly label a particular amino acid on an antibody. The ability to site-specifically introduce fluors into aptamers can potentially be exploited in signal transduction schemes. For example, many aptamers are known to undergo induced fit conformational changes upon interaction with their ligands (Padmanabhan et al., 1993; Burgstaller et al., 1995; Ye et al., 1999). We reasoned that upon ligand-binding an appended fluor might undergo a concomitant change in its chemical environment, leading to changes in fluorescence intensity, wavelength or anisotropy. To this end, we examined the known three-dimensional structures of several aptamers, including an anti-adenosine, RNA aptamer (Dieckmann et al., 1996; Jiang et al., 1996), an anti-adenosine, DNA aptamer (Lin and Patel, 1997), and an anti-arginine, DNA aptamer (Lin and Patel, 1996), and synthesized aptamers in which different fluorescent dyes were inserted adjacent to but hopefully not impinging on functional residues (Fig. 1a). The resultant 'signaling aptamers' were assayed for increases or decreases in the intensity of fluorescence in the presence of a cognate ligand.

Although not all signaling aptamers underwent ligand-dependent changes in fluorescence, some of the aptamers demonstrated quite good signaling characteristics. For example, the anti-adenosine, DNA signaling aptamer in which fluorescein was inserted between residues 7 and 8 could be used to directly and accurately quantitate the

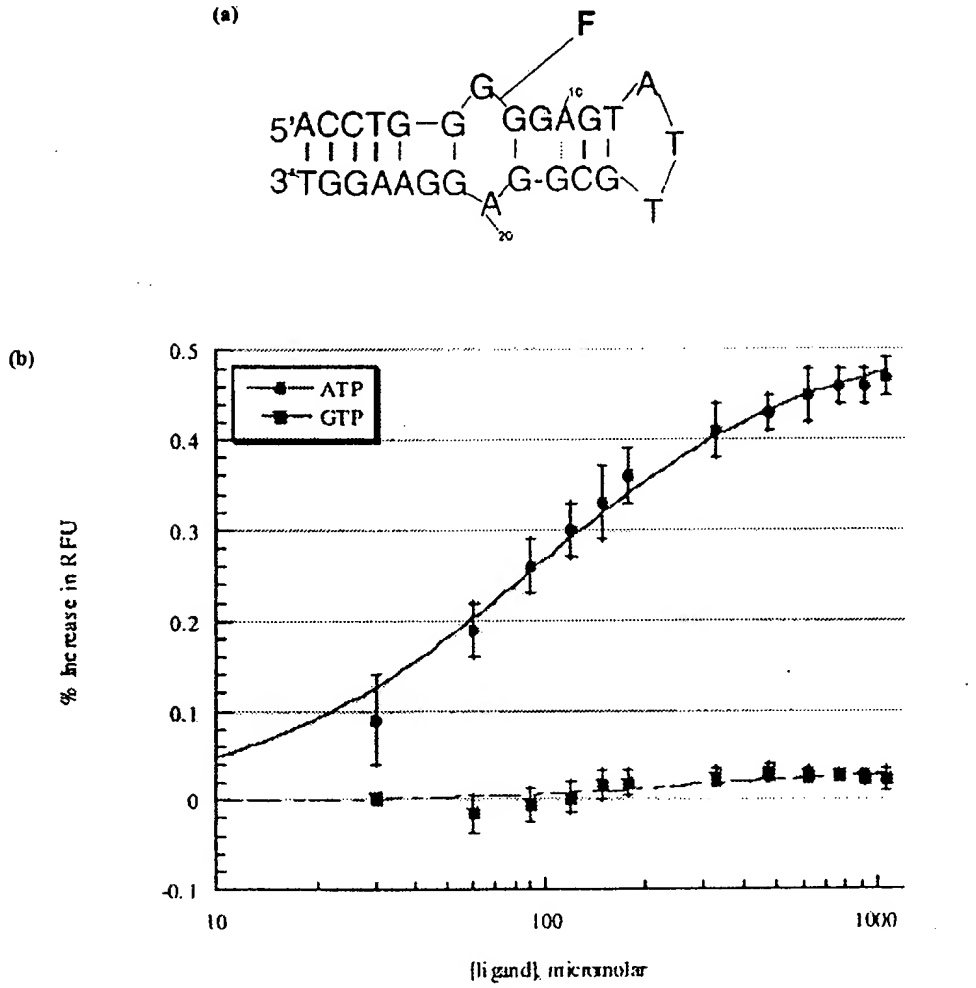

Fig. 1. (a) Secondary structure and signaling profile of the signaling aptamer DFL7-8. A fluorescein phosphoramidite was inserted in between residues 7 and 8. (b) The DNA aptamer shows a concentration-dependent increase in relative fluorescence for its cognate ligand, ATP, but not for a non-cognate ligand, GTP.

presence of ATP in solution (Fig. 1b). Just like the original anti-adenosine aptamer, the signaling aptamer can readily discriminate against non-cognate nucleotides, including GTP, UTP, and CTP.

Unfortunately, despite our best attempts at rational design, the anti-adenosine, DNA signaling aptamer did not sense ATP as well as the parent molecule from which it was derived bound to ATP. The introduction of the fluorophore is likely to have perturbed the proximal binding site. The signaling aptamer displayed a half-maximal change in relative fluorescence in the presence of 90 μM ATP, whereas the published K_d value for the ATP aptamer is approximately 6 μM. A similar loss of affinity was observed for several other signaling aptamers as well. While our results indicate that it might be possible to readily convert aptamer receptors to aptamer biosensors, the loss of binding affinity limited the potential utility and dynamic range of designed signaling aptamers. Therefore, we have more recently embarked on two additional, less rational methods to generate signaling aptamers.

First, we attempted the stochastic incorporation of fluoresceinated nucleotides into RNA aptamers. Fluorescent analogs, commercially avail-

able as nucleoside triphosphates, are readily incorporated into RNA. Varying the ratio of fluoresceinated to non-fluoresceinated nucleotides during transcription should result in populations of aptamers that carry varying numbers of fluorescent reporters, randomly distributed about the sequence and structure of the aptamer. We reasoned that some of the fluorescently labeled aptamers would show increased fluorescence in the presence of a cognate ligand, some would show a decrease, and many would be neutral or inactive. Overall, though, the population should have a net increase or decrease in fluorescence in the presence of the cognate ligand. As proof-of-principle, an anti-tobramycin aptamer was transcribed using ratios of 25:1 and 10:1 of UTP/F-12-UTP, and the resulting populations were assayed for changes in signaling in the presence of their cognate ligand, tobramycin, and a non-cognate aminoglycoside, lividomycin. While the 10:1 population displayed a 5% increase in relative fluorescence in the presence of its cognate ligand and no response to lividomycin, the 25:1 population showed no such increase. In fact, in the presence of lividomycin the 25:1 population showed a 5% decrease in relative fluorescence.

We are also attempting to select for fluorescent signaling aptamers ab initio. We reasoned that the pre-selection incorporation of the fluorescent reporter (as opposed to the post-selection incorporation previously attempted) should minimize any dissonance between tight binding and signaling. Pools of fluorescent RNA in which individual bases (e.g. U) have been completely substituted for by their fluoresceinated analogs (e.g. F-12-U) have been synthesized. In order to reduce the background fluorescence and maximize potential changes in relative fluorescence, the random sequence regions of these pools have been canted so that only a small number of fluoresceinated bases are on average incorporated into each RNA. The selection for signaling aptamers is being carried out in essentially the same way as a selection for tightly binding species. At the conclusion of the selection, the population as a whole and individual aptamers will be screened for ligand-dependent changes in fluorescence intensity.

Finally, we have developed a sensor system that can sensitively detect ligand-dependent changes in fluorescence anisotropy rather than fluorescence intensity (Potyrailo et al., 1998). An anti-thrombin, DNA aptamer was synthetically labeled with fluorescein at its 5′ end and with an alkyl amine at its 3′ end. The signaling aptamer was then immobilized on a glass surface, and protein-binding was monitored by surface plasma resonance spectroscopy. As little as 5 nM of protein could be detected in an inquired volume of 5 nl, suggesting that attomole amounts of protein could be specifically visualized. One of the advantages of this format was that the aptamer could be labeled distal from the ligand-binding site (as opposed to adjacent to the binding site, as in the examples cited above), since the change in anisotropy was dependent on the formation of the complex rather than on the specific nature of the aptamer:protein interactions. In addition, this format is particularly attractive as it involves the labeling of the receptor (aptamer) rather than of the analyte stream, potentially allowing real-time analysis of unlabelled samples.

4. Aptazymes

Just as induced fit and other conformational changes can be used to modulate the environment of a fluorescent reporter, the same ligand-dependent changes in state can be used to control ribozyme catalysis. Ron Breaker and his coworkers at Yale University originally showed that appending an anti-adenosine aptamer to the hammerhead ribozyme allowed RNA hydrolysis to be specifically modulated by adenosine (Tang and Breaker, 1997b, 1998; Soukup and Breaker, 1999a). The Breaker lab has since built upon this feat by randomizing the connecting region between the hammerhead and aptamer domains and selecting so-called 'communication modules' that facilitate ligand-dependent increases or decreases in catalytic activity (Soukup and Breaker, 1999b). Astoundingly, different aptamers can be conjoined with the hammerhead via these communication modules and correspondingly change which ligand controls catalysis.

Our lab has similarly pursued the development of allosteric ribozymes, which we term aptazymes. Originally, Michael Robertson used a scheme similar to that employed by Bartel and Szostak (1993) to select a novel ribozyme ligase from a pool that contained 90 random residues. The resultant ligase was relatively slow (single-turnover rate of 0.71/h), but proved to be extremely dependent on a cDNA primer for catalytic activity. In order to improve catalysis and better understand ligase structure and mechanism of action, we doped the ligase and selected active variants. The pattern of sequence change and conservation following selection were consistent with the ligase forming a three-helix junction in which residues adjacent to the junction were important for catalytic activity (Fig. 2). Interestingly, the ligase chose not to utilize a substrate-binding site that had been built into the original RNA pool, but instead created its own substrate-binding domain near its 3′ end, as had a number of previously selected ligases (Robertson and Ellington, 1999b). Based on the hypothesized structure, we were able to model how the presence or absence of the oligonucleotide effector (cDNA primer) regulated ribozyme catalysis. In the presence of the primer, the 3′ end of the ribozyme was occupied. In the absence of the primer, however, the 3′ end of the ribozyme folded over onto the substrate-binding site, blocking substrate-binding and catalysis, just as the pseudosubstrate domains of protein kinases frequently block substrate access and catalysis. This antisense mechanism of ribozyme regulation is similar to one proposed several years earlier by Porta and Lizardi (1995). However, the selected allosteric ribozyme was regulated over

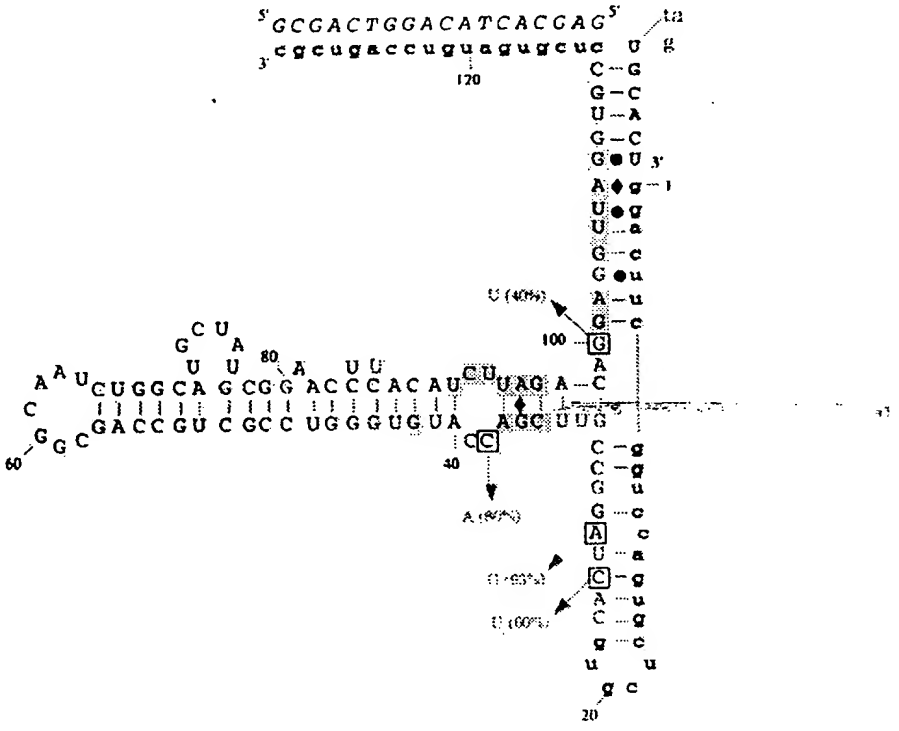

Fig. 2. Secondary structure of the L1 ribozyme deduced from a doped sequence selection. Nucleotides in lowercase red are constant sequence priming regions not mutagenized in the doped selection. Nucleotides in shaded boxes were absolutely conserved following re-selection, while nucleotides in blue were highly conserved (> 85%). Boxed positions converged to the unique, non-wild-type nucleotide indicated in green. The ligation substrate is shown in purple, and the oligonucleotide effector in italics.

four orders of magnitude by its cognate effector, while the previously designed allosteric ribozyme was regulated only 10-fold.

To the extent that our model for oligonucleotide activation was correct, it should be possible to rationally alter the effector specificity of the L1 ligase by altering the sequence of the 3′ primer-binding site. A series of variant ribozymes was prepared and assayed either in the presence of the wild-type (and hence mismatched) effector or in the presence of variant effectors that were predicted to be completely complementary. As expected, the variant ribozymes could not be efficiently activated by the wild-type effector, but did show activation in the presence of the appropriate complementary effectors. However, the level of activation with variant ribozyme and effector pairs was generally less than was originally observed with the wild-type ribozyme and effector. This was also expected. While the 'active' conformers of the variant ribozymes were stabilized by the variant effectors, their 'inactive' conformers (which relied on internal Watson–Crick pairing) were correspondingly destabilized.

Breaker and his co-workers had originally designed an effector-dependent hammerhead ribozyme by joining ribozyme residues and structures known to be important for function with aptamer residues and structures known to be aligned and stabilized by induced fit (Tang and Breaker, 1997a, 1998). Our selection and mutational analyses had been successful in identifying which residues in the L1 ligase were important for function. To further validate the structural and functional model of the L1 ligase that had emerged from mutation and selection analyses, we attempted to generate a minimal ribozyme by deleting residues in outlying portions of the ribozyme that appeared to be less critical to ribozyme function. In particular, Stem C extends outward from the helical junction with mostly Watson–Crick base-pairing interrupted by a single, absolutely conserved G:A pair and a symmetrical internal loop. However, beyond the internal loop the majority of stem C was highly variant following the doped selection and did not form a consensus secondary structure. The activity of the ribozyme proved to be quite refractive to deletion, retaining most of its activity even when most apparently superfluous residues were removed. Consistent with the doped selection data, constructs with deletions downstream of the A40:U91 base pair retained full activity, while constructs with deletions encompassing the C38C39:C92U93 internal loop were inactive.

Based on these results, we attempted to join several different aptamers to the L1 core structure in hopes that the hybrid aptazyme would exhibit effector-dependent ligation activity. The first aptazyme construct was created by introducing an anti-adenosine aptamer in place of the non-essential portion of Stem C. Since the minimal, functional structural model of the L1 ligase appeared to require the stem C internal loop region; the anti-adenosine aptamer was joined to a base pair immediately adjacent to the internal loop (Fig. 3). It was known from NMR studies that the anti-adenosine aptamer adopted a more rigid conformation upon interaction with adenosine. We hypothesized that upon interaction with adenosine, the connecting stem would be stabilized, would better mimic the Watson–Crick pairing normally present on stem C, and thus, would better align the catalytically important internal loop region. This hypothesis proved to be correct; the resultant aptazyme showed a 30-fold increase in activity in the presence of saturating ATP concentrations.

To the extent that the ligand-dependent stabilization of the joining region between the ribozyme and the aptamer led to ligand-dependent increases in catalysis, then destabilization of the joining region should similarly lead to an increased level of allosteric activation. We synthesized and assayed a series of constructs that rationally altered the joining region, progressively destabilizing the connecting stem structure by either introducing mismatches into or shortening the stem structure. Both destabilization approaches were successful in increasing the ATP aptazyme's activation parameters. The greatest activation (830-fold) was observed using a truncated stem B construct in which tandem U:G wobble pairs replaced the central U:A pairs of the connecting stem.

Based on the success of our ATP aptazyme, we used the same strategy to design additional new

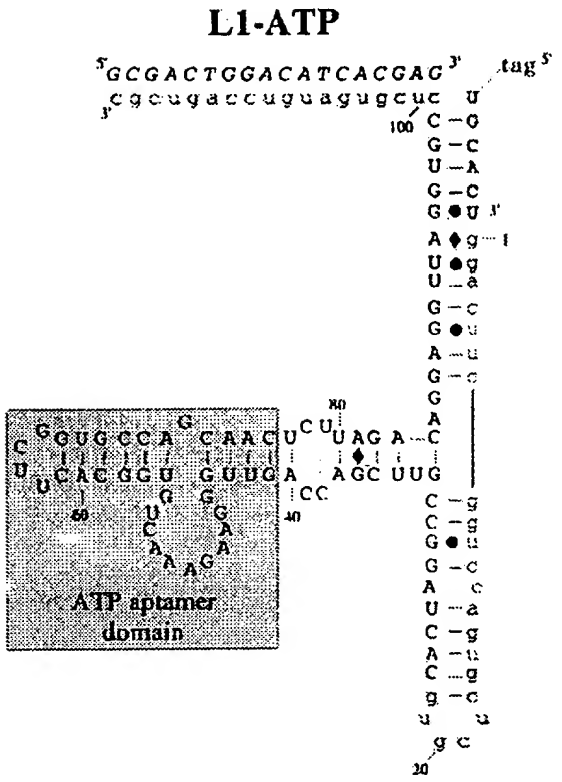

Fig. 3. ATP-dependent aptazyme ligase. The color scheme is as in Fig. 2, with the anti-ATP aptamer domain indicated by the shaded box. The activity of the aptazyme is increased 30-fold in the presence of saturating ATP.

aptazymes by fusing other types of aptamer domains onto stem C of L1. We chose to use anti-theophylline and anti-flavin aptamers, which had previously proven amenable for use in hammerhead aptazymes, in order to compare the characteristics of hydrolase and ligase aptazymes. The anti-theophylline aptamer was appended to stem C by a 4-bp connecting stem. This initial theophylline construct showed less than twofold activation with theophylline and so a new construct was designed in which the connecting stem was destabilized by replacing a U:A base pair with a U:G wobble pair. The activation parameters of the second generation construct were no better than the initial construct and so a third-generation construct was created in which the connecting stem was shortened to 3 bp by deleting a G:C base pair. This version of the aptazyme proved to be extremely dependent on the presence of theophylline for activity. Without theophylline, the ribozyme performed self-ligations with a rate of 1.7×10^{-4}/h. When theophylline was added to the reaction the rate improved to 0.27/h, a 1600-fold increase.

The anti-flavin aptamer was appended to stem C by a 4-bp connecting stem. Like the initial theophylline construct, the initial flavin construct showed little activation. However, in contrast to the results with the anti-adenosine and anti-theophylline aptamers, simple destabilization mutations of the connecting stem did not readily lead to improved activation parameters. In addition, we attempted to insert a 'communication module' that had previously been selected by to improve the FMN-dependent activation of a hammerhead aptazyme (Soukup and Breaker, 1999b). However, in the context of the L1 ligase the hammerhead communication module did not yield any effector-dependence; in fact, the resultant ligase construct was completely inactive. These results indicate that allosteric mechanisms are not generalizable and may have to be independently developed for each new aptazyme.

In order to optimize the performance of the anti-flavin and anti-chloramphenicol aptazymes, the stem region connecting the aptamer and ribozyme domains was randomized and a selection for effector dependence was performed. The randomized pools were synthesized such that each side of the putative stem contained a random sequence mixture either three or four nucleotides in length. A two-stage selection procedure was used to isolate aptazymes that achieved maximal activity only when ligand was present. The first, negative, selection step involved an extended incubation with substrate in the absence of ligand. Any ribozymes capable of ligating without ligand being present were removed from the population via a biotin tag on the substrate. Ligand was then added to the remaining RNA population and a second, positive, selective step was used to isolate the best ligators from the surviving ribozymes. The entire process was repeated in an iterative fashion with an increasingly stringent positive se-

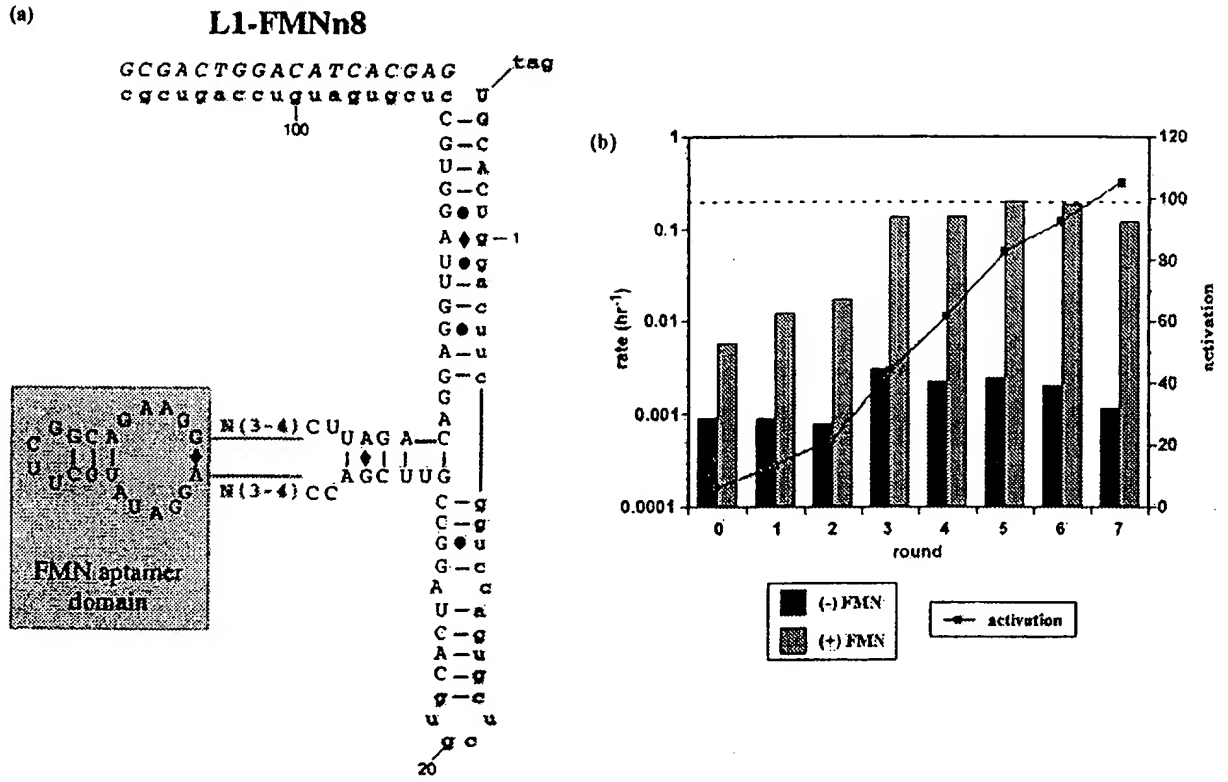

Fig. 4. Optimization of an FMN-dependent aptazyme ligase. (a) Random sequence pool for selection. The color scheme is as in Fig. 2, except that the random sequence region is shown in green. The anti-flavin aptamer domain is indicated by the shaded box. (b) Blue bars indicate the reaction rate in the absence of ligand and green bars indicate the rate in the presence of FMN. The red line shows the level of activation in the presence of saturating FMN that was achieved for each round of selection.

lective step until only the most active aptazymes remained. Over the course of seven rounds of selection for FMN activation, the activity of the selected population generally increased when FMN was present, while the activity of the same population in the absence of FMN remained low (Fig. 4). The net result was a population of aptazymes that reacted at rates comparable to the wild-type L1 ligase when FMN was present, but approximately 100-fold slower in the absence of FMN. When individual clones were isolated from the selected population and assayed for activity, most variants had activations of approximately 100-fold with FMN in accord with the activity level of the population. However, a few variants were isolated that had superior activation parameters of up to 250-fold with FMN.

5. High-throughput construction of chips to sense proteomes and metabolomes

Aptamers have already been selected against an extremely wide range of analytes. Since aptazymes can be generated by conjoining aptamers and ribozymes, it should be possible to produce aptazymes against a similar range of analytes. To facilitate the high-throughput generation of nucleic acid receptors and sensors against a wide range of targets, we have recently automated the in vitro selection procedure (Cox et al., 1998).

In particular, in order to readily generate aptamers that can recognize numerous protein targets, perhaps even a large fraction of an organismal proteome, we are attempting to carefully determine how epitopes on protein targets are

recognized by aptamers. We have previously selected aptamers to a variety of arginine-rich motifs found on viral proteins (Baskerville et al., 1995, 1999; Srinivasan et al., 1996; Ye et al., 1996, 1999), and in collaboration with Dinshaw Patel at the Memorial Sloan-Kettering Cancer Center have determined the structures of aptamer: peptide complexes. Surprisingly, it appears as though aptamers form binding cusps that can alter the conformation of their peptide targets. For example, an anti-Rev ARM aptamer selected against an alpha helical conformer of the ARM can induce alpha helicity, while an anti-Rev ARM aptamer selected against a random coil conformer of the ARM can reduce alpha helicity (Ye et al., 1999). These results provide the likely rationale behind our previous findings that aptamers selected against peptide targets can cross-recognize their cognate proteins (Xu and Ellington, 1996; Blind et al., 1999), just as anti-peptide antibodies can frequently recognize the same peptide epitope within the context of a full-length protein. To the extent that we can identify which peptides are most likely to elicit aptamers and determine which anti-peptide aptamers are most likely to cross-react with protein targets, we can potentially scan organismal genomes for appropriate peptide sequences, quickly synthesize the peptides in parallel, and then select aptamers that will map throughout the length and breadth of a genome/proteome.

Both aptamers and aptazymes can be readily modified to generate signals. In the case of aptamers, we have seen how either the stochastic incorporation of fluorescent nucleotides or the pre-selection incorporation of fluorescent nucleotides can directly lead to aptamers that signal interactions with their targets. Either of these techniques can be applied to multiple aptamers in parallel. Aptazymes are even more conducive to inclusion in signal transduction schemes. The aptazyme ligases we have developed could, for example, ligate an oligonucleotide containing a fluorescent dye to themselves. Indeed, the ligases could potentially append any of a number of reporter tags to themselves, including oligonucleotides coupled to enzymes that turnover fluorescent substrates, such as beta-galactosidase, oligonucleotides coupled to enzymes that can generate electrons for electrochemical sensing, such as horseradish peroxidase.

Since aptamers and aptazymes can be synthesized with pendant chemical moieties it should be possible to covalently immobilize multiple, different aptamers in discrete sectors of arrays. For example, a host of signaling aptamers could be synthesized with terminal amines, immobilized on glass, and an analyte mixture could be applied to the glass surface. The presence and quantities of individual analytes could then be determined by monitoring the changes in fluorescence intensity in individual sectors of the chip. Similarly, aptazymes could be immobilized and analytes and oligonucleotide tags introduced together. Since the pairing between the aptazymes and their oligonucleotide tags can be altered at will (see above), analytes could activate specific aptamers in specific sectors to pull down specific tags. In this way, analyte detection might be not only spatially but also spectrally resolved. Moreover, because the tags are covalently immobilized to the aptazyme, which is in turn covalently immobilized to the chip surface, aptazyme chips can be stringently washed to reduce non-specific binding and background. We are currently working towards proofs-of-principle for such arrays.

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Automated RNA Selection

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In vitro selection can be used to generate nucleic acid ligands (aptamers) to target molecules ranging in size and structure from cations to cells. However, the selection process is repetitive and time-consuming. We have automated a protocol for in vitro selection using an augmented Beckman Biomek 2000 pipetting robot. The automated selection procedure requires the integration of four devices and the optimization of four molecular biology methods, and is one of the most complex automated protocols attempted to date. Initial attempts at selection yielded robust replication parasites, but optimization of the automated selection procedure suppressed the emergence of these parasites and led to the selection of true nucleic acid ligands. Automated selection can now be used to generate nucleic acid aptamers in days rather than weeks or months.

Introduction

A number of laborious tasks in molecular biology such as plasmid preparation, DNA sequencing, and microarray construction and processing have been automated (*1*–*7*). The automation of these procedures has typically resulted in a substantial increase in the throughput for sample preparations or assays and a decrease in the amount of time an experimenter must devote to purely mechanical manipulations.

We and others have previously shown that nucleic acid binding species (aptamers) that interact tightly and specifically with a variety of ligands, from small organic molecules to supramolecular structures, can be selected from random sequence pools (*8*, *9*). The manipulations involved in in vitro selection experiments must be carried out over a number of rounds and can prove to be extremely time-consuming. For example, a single round of a selection experiment requires the preparation and purification of RNA, the filtration of RNA:target complexes, and the reverse transcription and PCR amplification of selected species. Given that from 5–20 rounds of selection are typically required to generate aptamers or catalysts, most in vitro selection experiments take from weeks to months to complete. Moreover, in contrast to other experimental procedures, there is not necessarily an economy-of-scale associated with multiple selection experiments against multiple targets, since the multiple, manual manipulations required for each step cannot usually be combined and must instead themselves be multiplied for each new target. However, the manipulations involved in in vitro selection experiments are similar to those in many other automated laboratory protocols. We have therefore attempted to automate a typical in vitro selection protocol using a specially modified Beckman Biomek 2000.

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Materials and Methods

Materials. The double-stranded DNA and RNA libraries were generated as previously described (*9*) and are shown in Figure 2. The primers used for amplification were "41.30", GATAATACGACTCACTATAGGGAATGGATCCACATCTACGA (T7 RNA polymerase promoter underlined), and "24.30", AAGCTTCGTCAAGTCTGCAGTGAA. Magnetic beads conjugated to $(dT)_{25}$ were obtained from Dynal (Lake Success, NY). Barrier pipetting tips (Beckman, Fullerton, CA) were used for all liquid transfers. The 96-well microplates (Falcon 3911 Microtest III flexible assay plates) were from Becton Dickinson (Oxnard, CA), except for those used in the thermal cycler, which were MJ Multiplate 96 microplates (MJ Research, Watertown, MA).

Robotics. All liquid and mechanical manipulations were carried out by using the Beckman Biomek 2000. The Biomek was interfaced with a PTC-200 thermal cycler (MJ Research), a magnetic bead separator (MPC-auto96, Dynal), and a Peltier cooler of our own design via a Dimension XPS H266 computer (Dell, Round Rock, TX). The schematics for the Peltier cooler are available on request. These devices and their relative orientations are shown in Figure 1a.

Robot Configuration. The worksurface of the Biomek 2000 was configured as shown in Figure 1b. The Peltier cooler ("enzyme cooler" in Figure 1b) held enzymes for the amplification steps; all other reagents for the selection, such as the binding buffer, were adjacent to the cooler in a microplate ("reagent tray"). To prevent evaporation of reagents from the microplate trays over extended periods of time, the plates were sealed with Beckman Sample and Seal Lids, thin adhesive sheets of aluminum foil that the Biomek can easily puncture with its pipet tip when aspirating or dispensing a new liquid. Bulk quantities of wash buffers were kept in a Biomek modular reservoir ("wash buffers"). Tips were sterilized prior to being loaded on the robot.

Software. The program directing the Biomek's movements was written mostly in BioWorks version 2.2, with small supplemental BioScript Pro library scripts. Com-

10.1021/bp980097h CCC: $15.00
Published on Web 10/29/1998

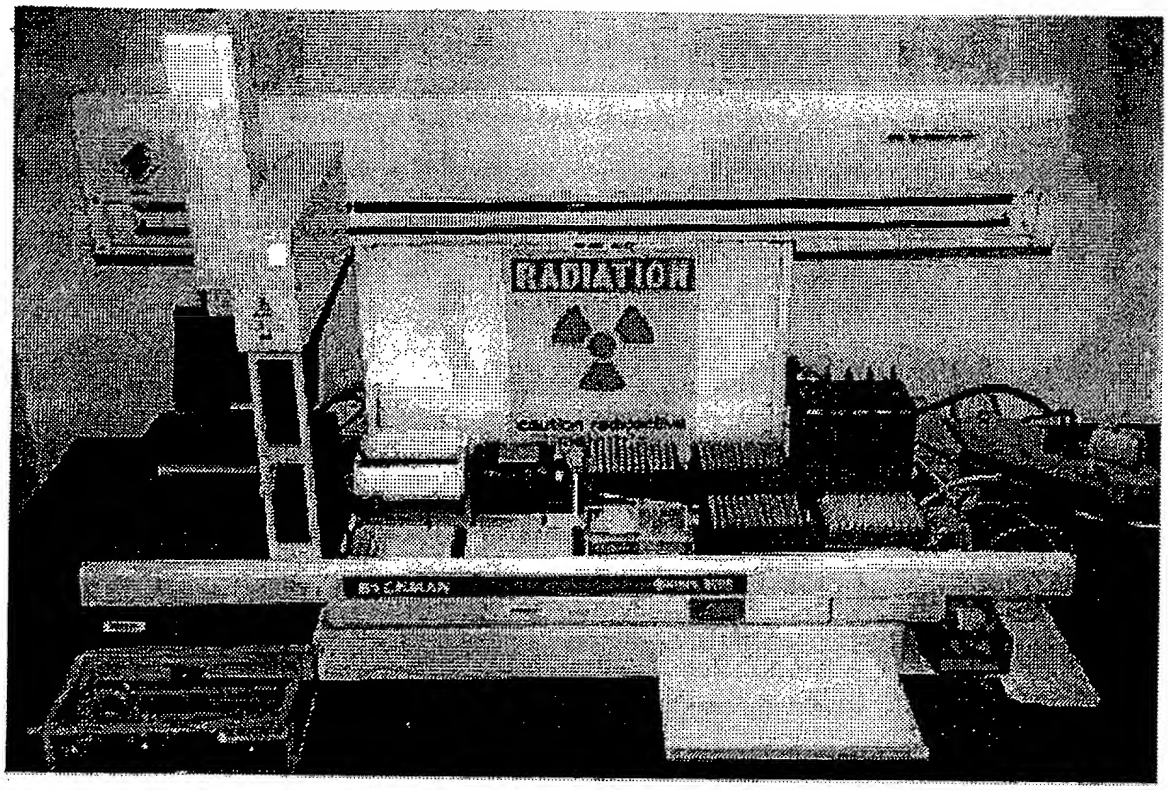

Figure 1. Selection robot. (a, top) The augmented Biomek 2000 used for automated selection. As landmarks, the PTC-200 thermal cycler is on the upper left, while the pipet tools are on the upper right. Radioactive tips from the experiment were discarded onto a sheet of plastic wrap covering the PTC-200 and dropped into a radioactive waste container at the lower left. (b, bottom) Schematic of the $x-y$ plane of the robot. The functions associated with the various positions are described in the text.

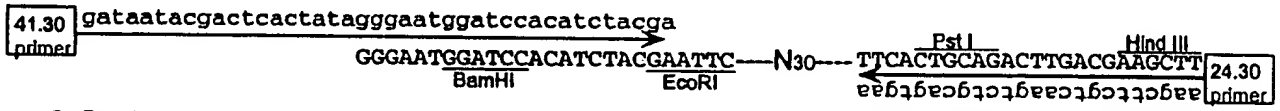

Figure 2. Random sequence pool. The N30 pool has previously been described (*9*). The random sequence core is flanked by constant sequences required for enzymatic amplification. Residues that will be found in the transcribed RNA pool are capitalized; primer sequences are in lower case. The primer containing the T7 RNA polymerase promoter is at the left, while the primer containing the reverse transcription start site is at the right. Restriction sites that facilitate cloning are indicated.

munication between the MJ Research PTC-200 and the Biomek was mediated by a serial connection between the thermal cycler and the computer and by Jeff Cahlik's (Beckman Coulter) MJR Biomek 2000 Driver. While the MJR thermal cycler could also have been directly interfaced with one of the Biomek's connection ports, Cahlik's driver allowed suspension of the BioWorks program until the completion of the thermal cycler's run. This obviated the problem of having to guess how long the thermal cycler might take to complete its program. The program for automated selection is available on request.

Selection Regime. At the beginning of the experiment, Dynabeads Oligo(dT)$_{25}$ (20 μL, corresponding to 6.6 $\times$ 10^6 beads) in a high-salt binding solution (80 μL; 20 mM Tris-HCl (pH 7.5), 1.0 M LiCl, and 2 mM EDTA) were loaded into a well in a microplate on the magnetic bead separator. Roughly 1 μg ($\sim 10^{13}$ sequences) of a random sequence RNA library (N30; 80 μL in transcription buffer, see below) was added to the beads. The binding reaction was thoroughly mixed by 10 cycles of rapid aspiration and dispensing. The binding reaction was incubated at room temperature for 2.5 min, mixed again (10 cycles), and incubated an additional 2.5 min. At the end of incubation, the MPC-auto96 raised magnets between the rows of wells in the microplate and captured the magnetic beads along the side of the well. The supernatant was removed (less than 0.5 μL typically left behind). The magnets were then lowered, and the beads

were resuspended and washed with 100 μL of low-salt wash buffer (10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, and 1 mM EDTA) to remove nonspecifically binding nucleic acid species. As before, the beads and buffer were mixed by 10 cycles of aspiration and dispensing. The wash step was repeated once more before resuspending the beads in 53 μL of water. The bead slurry was transferred to a well in the microplate in the PTC-200 thermal cycler, the lid of the machine was closed, and the slurry was heated to 65 °C for 3 min to dissociate nucleic acid binding species from the derivatized beads. During the elution, the lid of the thermal cycler was kept at 80 °C. After the elution step, the magnetic bead mixture was transferred back to the magnetic particle separator to ensure the complete removal of magnetic beads from the eluate. To amplify the selected RNA molecules, the eluate (51 μL, purposefully leaving behind a small amount of liquid) was transferred to a new well of the microplate on the thermal cycler.

Reverse Transcription and PCR. Conversion of the selected RNAs into double-stranded DNAs was carried out in a single step. RT-PCR reaction buffer (45 μL) was added to the 51 μL of selected RNA eluate. Upon mixing and dilution the amplification reaction contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5% acetamide, 0.05% Nonidet P40, and 0.5 μM each of 5′ and 3′ primers. To follow the progress of the selection, 5 μCi of α-^{32}P-labeled dATP was also included in the amplification reaction. The reaction mixture was incubated at 65 °C for 10 min (lid = 80 °C) to facilitate denaturation of secondary structures present in the selected RNAs that could inhibit reverse transcription. Finally, the temperature of the reaction was reduced to 50 °C, and 4 μL of RT-PCR enzyme solution was added to the prewarmed mixture. The RT-PCR enzyme solution contained 0.2 units of Display Taq (Promega, Madison, WI) and 5 units of AMV reverse transcriptase (RT) (Amersham Pharmacia Biotech, Arlington Heights, IL). To increase the volume of the enzyme mixture to 4 μL, these enzymes were diluted into a solution containing 50% glycerol, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$. Enzymes were not diluted until the start of the experiment and were kept in the enzyme cooler (−20 °C). The addition of the enzyme mixture brought the final volume of the RT-PCR reaction to 100 μL. Reverse transcription proceeded at 50 °C for 10 min, and the amplification reaction was then thermally cycled (45 s at 94 °C, 60 s at 50 °C, and 90 s at 72 °C) seven times. At the end of the RT-PCR reaction, 10 μL (one-tenth) of the products was transferred to a new well of the microplate on the thermal cycler.

In Vitro Transcription. Transcription reaction buffer (87 μL) was added to the newly amplified DNA templates. Upon mixing and dilution, the transcription reaction contained 40 mM Tris-HCl (pH 7.9), 26 mM $MgCl_2$, 0.01% Triton X-100, 2.5 mM spermidine, 5 mM dithiothreitol, and 2.5 mM of each NTP. To follow the progress of the selection, 5 μCi of α-^{32}P-labeled UTP was included in the transcription reaction. Transcription was initiated by the addition of 100 units of T7 RNA polymerase (Stratagene, La Jolla, CA) and 40 units of RNasin (Promega) in a total volume of 3 μL. The transcription reaction was allowed to proceed at 37 °C for 75 min.

Additional Rounds of Selection. A majority of the transcription reaction (80 μL) was transferred to a well on the magnetic particle separator that contained magnetic beads (6.6 × 10^6 beads) in the high-salt buffer (80 μL). The binding reaction was mixed as described above, and separation of bound and unbound RNAs proceeded as before. The entire selection and amplification procedure was repeated without change for four additional rounds.

Sequencing. At the end of the five cycles of selection and amplification, 1 μL of the final transcription reaction was used to seed 100 μL of a RT-PCR reaction similar to those described above. Double-stranded products were cloned into pCR2.1 with Invitrogen's (Carlsbad, CA) TA cloning kit according to the manufacturer's instructions. Individual colonies were screened for the presence of insert using a colony PCR protocol (*10*). Purified plasmids containing inserts were then sequenced using Epicentre's (Madison, WI) Sequitherm Excel II DNA sequencing kit according to the manufacturer's directions. Because long runs of a single base were expected, the isothermal (rather than thermal cycle) sequencing reaction directions supplied with the kit were employed.

Results and Conclusion

Design of the Automated Selection Station. The Beckman Biomek 2000 is an automated pipetting robot that can manipulate liquids in a limited $x-y-z$ volume. To satisfy all of the requirements for a typical in vitro selection experiment, we augmented the Biomek 2000 with a magnetic bead separator, the Dynal MPC-auto96 (to sieve target:nucleic acid complexes from free nucleic acids), and an MJ Research PTC-200 PCR machine (for nucleic acid amplification). In addition, we built a small Peltier cooler to hold enzyme solutions necessary for amplification. The augmented robot is shown in Figure 1a, and the layout of the $x-y$ plane for selection experiments is shown in Figure 1b. It should be noted that the $x-y$ grid has been laid out to avoid cross-contamination problems that might arise between parallel or serial selection experiments: "clean" tips that have not seen amplicons always move from right to left on the surface, eventually being discarded at the far left. Other precautions against cross-contamination include the use of barrier tips and puncturable seals on microplates.

Selection Experiments. A brief description of the automated selection procedure follows; a more complete description can be found in Materials and Methods. A random sequence RNA pool (N30; Figure 2) that had previously yielded aptamers (*9*) was used as a starting point for selection. The pool was loaded into a well in the "reagent" microplate. The Biomek transferred a portion of the randomized RNA pool (1 μg, ~10^{13} different sequences) into a solution containing magnetic beads derivatized with a target molecule, in this case $(dT)_{25}$. Oligo(dT) was chosen as a target to evaluate the efficiency of the robot and to facilitate interpretation of the results of the automated selection. The binding reaction was pipetted up and down several times to ensure adequate mixing of pool and target, and then incubated for 5 min at room temperature to allow nucleic acid:target complexes to form (total time = 7 min). The magnetic beads and associated RNA molecules were captured using a magnetic particle separator, and unbound RNAs were removed. To ensure that only high-affinity species were retained, the beads were then washed several times with a low salt solution and the washes removed (10 min). Any remaining RNA molecules were eluted from the target by the addition of a no-salt (water) solution. To ensure efficient elution, the beads were transferred to the thermal cycler, and the elution step was carried out at 65 °C (10 min). All of the eluted species were used to seed a RT-PCR reaction; the selected population, buffers and substrates, and an enzyme mixture were separately added and mixed by the Biomek (110 min). One-tenth

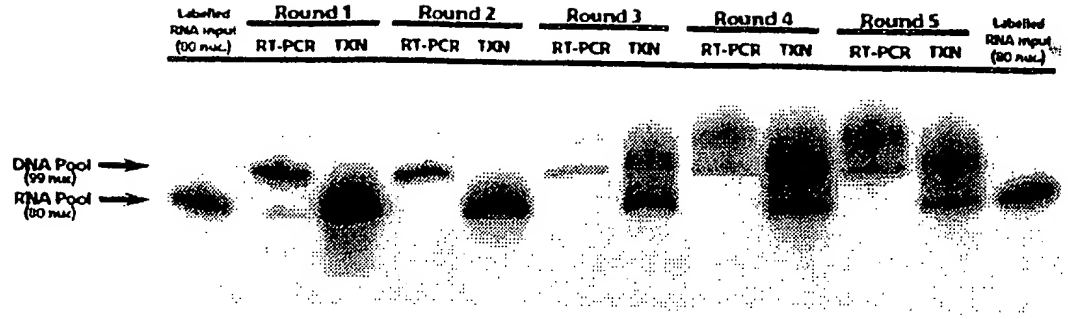

Figure 3. Progress of automated selection. RNA molecules corresponding in size to the N30 pool are shown at the left and right; the sizes of the RNA pool and DNA pool are indicated with arrows. A fraction (5 μL; 5%) of each RT-PCR ("RT-PCR") or in vitro transcription ("TXN") reaction was pipetted into separate wells in a microplate during the selection. The wells contained an equal volume of sequencing stop dye. At the conclusion of the selection, the samples were electrophoresed on a 8% denaturing polyacrylamide gel, the gel was exposed to a Phosphor Screen, and the screen was read using a Phosphorimager SI (Molecular Dynamics, Sunnyvale, CA).

Clone	Sequence	Longest adenosine run length	Total random length
FYL03	GCGATAAGTATCACACTTCGTTAAAAAAAAAAAAAAAAAAAAAAAAAG	25	48
FYL04	GCTTACTAGGAATACAAGCTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTTG	39	64
FYL08	TGGGGGGTAGGCTATCTGGGAAAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATGG	37	66
FYL09	TGCACGATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACGAGAACCCACAACTT	38	63
FYL13	AATGGACCGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATAAAGGTATA	31	51
FYL17	GCCACAGGAAAGGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGAGAAACG	29	51
FYL18	CAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACAGCGTTAATAAATACATATAAG	30	54
FYL20	TGGAGAAAGGTCGTAAAAAAAAAAAAAAAAAAAAAAAAGCACGTATT	21	44

Figure 4. Sequences of selected clones. Individual clones were isolated and sequenced as described in Materials and Methods. The sequences of the random sequence regions from these clones are shown. The adenosine run length and total length of the random sequence tract are shown at the right.

of the amplified template was then used for in vitro transcription (75 min). Finally, the amplified RNA, still in its transcription buffer, was introduced into a new round of selection. The total time required for a round of selection was 212 min.

Five rounds of automated selection were carried out using this procedure. To follow the production of double-stranded DNA templates and single-stranded RNA transcripts, small amounts of radiolabeled nucleotides (α-^{32}P dATP for DNA and α-^{32}P UTP for RNA) were included in the amplification reactions. A portion of each RT-PCR and transcription reaction was analyzed by gel electrophoresis (Figure 3). As can be seen, the robot initially faithfully reproduced the sizes of the DNA and RNA products, despite the fact that a constant number of thermal cycles had been programmed into each round of automated amplification. We had anticipated this result, since the N30 pool was designed to undergo many rounds of amplification without the production of artifacts (*11*) and had, in fact, previously yielded no replication parasites during selection experiments. However, by the third round of selection, a higher molecular weight species appeared and became even more prominent in both RNA and DNA populations in rounds 4 and 5. We initially assumed that the size heterogeneity was the result of imprecise automated amplification that had previously not been observed during manual amplification; however, these bands are, in fact, the result of a selected expansion of the random sequence region (see below).

Sequences of Selected RNA Molecules. Amplified RNA molecules from the last round of selection were further amplified using a RT-PCR protocol similar to that employed during robotic selection. The amplified, double-stranded DNA molecules were cloned, individual clones were screened for the presence of inserts using a standard colony PCR protocol (*10*), and clones containing inserts were sequenced. The sequences of several individual clones are shown in Figure 4. As expected, the success of the selection experiment was immediately apparent: each clone contained long, uninterrupted runs of adenosine. It is anticipated that $(dT)_{25}$ can readily be employed as a positive control in future automated selection experiments.

The sequences of the selected clones revealed the provenance of the larger molecular weight bands observed in rounds 3–5. The random sequence tracts had expanded to include 44–66 residues. The expansion appeared to be due primarily to an expansion of the target binding site, since runs of 21–39 contiguous adenosine residues were present. The runs were not preferentially localized within the transcripts and could occur at either end of the original random sequence tract. No shorter clones were obtained, likely because the majority of clones had become elongated by the conclusion of the selection (Figure 3).

The expansion of the random sequence region to include additional adenosine residues had previously been observed in manual selection experiments and was likely due to "stuttering" in the register of T7 RNA polymerase and/or reverse transcriptase as homopolymer tracts were copied (*12, 13*). However, the fact that such long runs or adenosines are produced so soon during the course of the selection experiment indicates that the selection procedure employed was quite stringent yet consistently yielded enough selected molecules for additional amplification. While molecules such as proteins will undoubtedly prove to be more difficult selection targets, these results bode well for the general application of the automated selection procedures.

Parameters That Influence the Success of Automated Selection Experiments. During the develop-

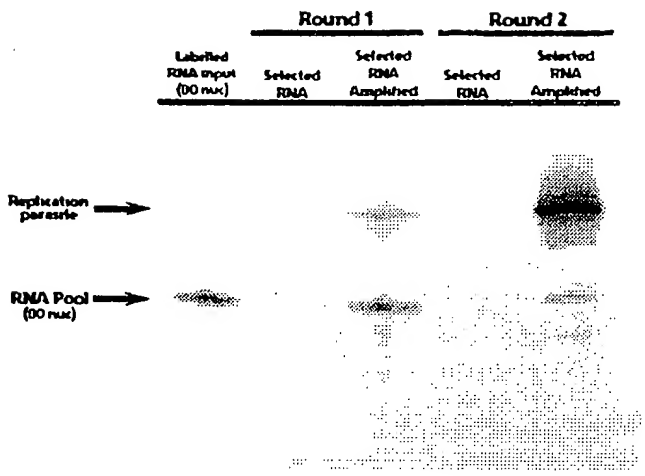

Figure 5. Isothermal amplification yields replication parasites. Experiments similar to those described in Materials and Methods were carried out, except that the 3SR procedure of Guatelli et al. (*14*) was used for amplification rather than RT-PCR followed by in vitro transcription. Fractions (5 μL; 5%) of the selected RNAs ("selected RNA") and isothermal amplification reactions ("selected RNA amplified") were run on an 8% denaturing gel. In the gel that is shown, only RNA molecules were labeled. No bands are seen in the "selected RNA" lanes, because early in the selection few RNA molecules are extracted from the population by the Dynabeads. The relative sizes of the RNA pool and the replication parasite are indicated by arrows.

ment of the automated selection method described above several variations were introduced. The amount or proportion of selected RNA that was introduced into amplification reactions for subsequent cycles of selection did not appear to influence the course of the selection, nor did the amount or proportion of newly transcribed RNA that was used for selection. The amount of enzymes used for amplification could be varied by factors of 2−3 without any observable changes in the dynamics of the selection experiment. The apparent robustness of the automated protocol to these modifications was especially encouraging, given that several different aspects of selection procedures (e.g., the amount of nucleic acid population bound in each cycle, the number of cycles of amplification necessary to obtain a PCR product, the amount of RNA obtained from transcription) are typically closely monitored by experimentalists during manual selection protocols. Overall, it appears as though these and other variables can be subsumed within common mechanized manipulations without significantly altering or belaying the progress of the selection.

However, one parameter that had a decided effect on the success of the selection experiments was the amplification method that was employed. We originally attempted to perform automated selection using an isothermal amplification procedure (3SR) that combined reverse transcription and transcription in one tube (*14*). The successful implementation of this procedure would have allowed at least one step to be excised from the protocol and would have sped up the turnaround time for each round of selection. However, attempts to amplify random sequence RNA molecules using the isothermal amplification protocol consistently led to the generation of amplification parasites that overran the selected RNAs (see, for example, Figure 5). The amplification parasites that were observed were themselves interesting, given that they were larger than the expected product, rather than smaller as are expected of amplification parasites, and have been further characterized. In contrast to the larger bands observed during a successful selection experiment (Figure 5), the parasites were quasispecies centered on a single molecule, did not contain runs of adenosines, and arose irrespective of whether oligo(dT)$_{25}$ was included in the automated selection protocol or whether the selections were carried out manually or by the robot. These results are especially significant given that previous selection experiments carried out with partially (as opposed to completely) randomized populations successfully employed 3SR as an amplification procedure (*15*). Unfortunately, while partially randomized nucleic acid populations centered on a particular molecular species generally reproduce faithfully, completely randomized populations offer many more potential routes to replication parasites. The likely success of "discontinuous" amplification procedures (RT-PCR followed by transcription) for molecular engineering relative to "continuous" amplification procedures (3SR) had previously been predicted by evolutionary biologists on theoretical grounds alone (*16*).

Prospects for Automated Selection. Just as in vitro selection techniques have been applied to a wide variety of targets from small molecules to proteins to supramolecular structures such as viruses and tissues, it should be possible to apply automated selection procedures to a similar range of targets. For example, Eigen and co-workers have previously built a PCR machine coupled with a liquid delivery system that allowed the parallel amplification and serial transfer of multiple nucleic acid samples (*17*). The automated protocol we have developed was designed to be carried out with off-the-shelf robotics and to be readily adaptable to a variety of selection modalities and conditions. A number of different derivatization chemistries can be used to conjugate target molecules to magnetic beads, and the amplification protocols are amenable to double-stranded DNA libraries, single-stranded DNA libraries, and modified nucleic acid libraries, as well as conventional RNA libraries of any length.

Using the duration of the current experiments as a guide, it should be possible to carry out at least 10 rounds/day of automated selection against a single target. By using multipipettor tools (e.g., the Beckman MP200), it should be possible to simultaneously select against 8−96 or more different targets or to assess 8−96 different selection conditions.

A final, often unappreciated, advantage of automated procedures relative to their manual counterparts is the consistency of repetitive tasks. In vitro selection experiments have previously been used to address evolutionary questions, but the relevance and uniformity of the data sets that have been derived have been open to question because there is large variation between how individual researchers or laboratories carry out the plethora of manual procedures that are required to extract "winning" sequences from random sequence libraries. Automated selection procedures should provide a reproducible benchmark for comparison, and may allow results from many different labs to ultimately be combined and interpreted.

Acknowledgment

This work was supported by NIH Grant No. AI 36083-03.

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Accepted October 7, 1998.

BP980097H

Express Mail No: EV312711638US
Date of Deposit: October 27, 2004

Attorney Docket No: 23239-301B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Ellington, *et al.*		
SERIAL NUMBER:	09/666,870	EXAMINER:	Jon D. Epperson, Ph.D.
FILING DATE:	September 20, 2000	ART UNIT:	1639
FOR:	METHOD AND APPARATUS FOR IDENTIFYING ALLOSTERICALLY REGULATED RIBOZYMES		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL DECLARATION TO FEBRUARY 11, 2004 DECLARATION OF PRIOR INVENTION UNDER 37 C.F.R. § 1.131

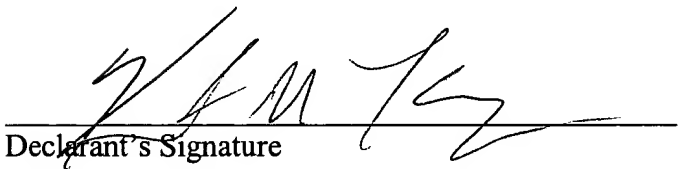
I, Kristin Thompson, hereby declare and state as follows:

1. The previous Declarations of Prior Invention Under 37 C.F.R. §1.131 filed on February 11, 2004 (the "February 2004 Declaration") inadvertently stated that I was a co-inventor of the inventions claimed in the above-identified application. For many years, I worked closely with the inventors of the subject matter recited by the claims in the instant application while I was a graduate student in Dr. Andrew Ellington's laboratory at the University of Texas at Austin. Dr. Ellington is the Principal Investigator of the laboratory where the inventions described in the instant Application were invented.

2. The instant application claims priority to a provisional application on which I am a named inventor, U.S.S.N. 60/212,097, filed June 15, 2000. Several applications claiming priority to this provisional application were filed with the U.S. Patent and Trademark Office and with the World Intellectual Property Organization, many of which name me as an inventor. A chart showing the inventorship for the progeny of this provisional application is attached hereto. In addition, I am the lead author of one of the references cited by the Examiner: Marshall and Ellington, Nature Structural Biology, 6(11):992-94 (1999) ("the Marshall reference").

3. At the time I executed the February 2004 Declaration I mistakenly believed that my contribution to the Marshall Reference was part of the subject matter of one or more claims of the present application. Thus, I inadvertently identified myself as an inventor in the February 2004 Declaration. This mistake was unintentional.

3. As a person signing below, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Declarant's Signature

9/24/04

Date

Full Name of Declarant: Kristin Thompson

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